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Bioassay-Directed Chemical Analysis in Environmental Research

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16. ABSTRACT

It became apparent in the lat[illegible]s that genetic bioassays could be used in combination with chemical fractionat[illegible]greatly simplify the process of identifying significant mutagens in complex environmental samples such as diesel particles, ambient air particles, complex fuels, woodsmoke, industrial effluents, chemicals in drinking water, and commercial products. The use of short-term bioassay tests in conjunction with analytical measurements, constitute a powerful tool for identifying important environmental contaminants. We have coined the terminology "bioassay directed chemical analysis" to best describe this marriage of analytical chemistry and biology. The objective of this methodology is to identify key compounds in various types of air pollutant samples. Once that task is completed, studies on metabolism, sources, environmental exposure and atmospheric chemistry can be undertaken. The principles and methodologies for "bioassay directed chemical analysis" are presented and illustrated in this paper. Most of our work has been directed toward the characterization of ambient air and diesel particulates which we will use as examples in this report to illustrate the analytical logic used for identifying the bio-active components of complex mixtures.

17. KEY WORDS AND DOCUMENT ANALYSIS

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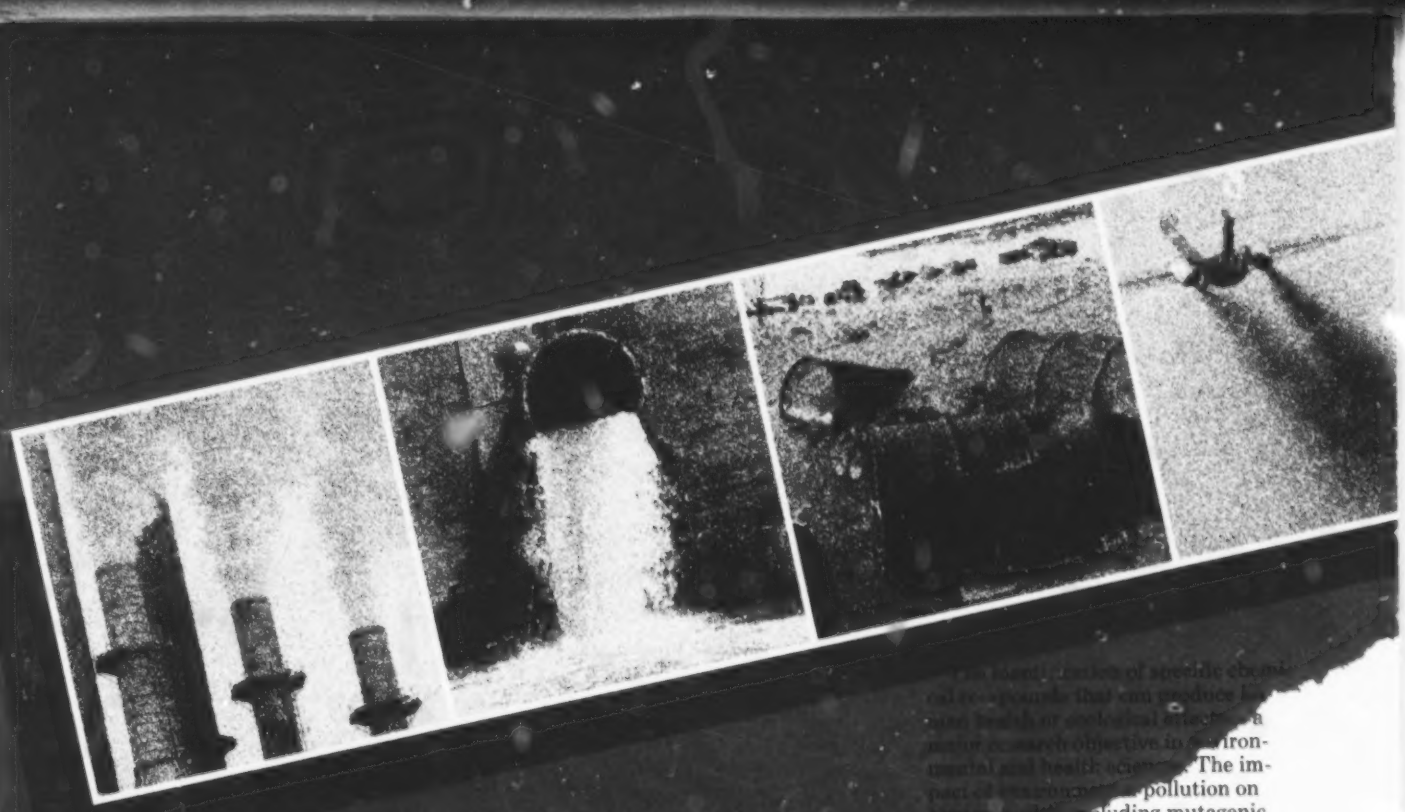

Bioassay-Directed Chemical Analysis in Environmental Reseach

...ironmental and health sciences. The impact of ... pollution on ... including mutagenic and ...genic effects, is a primary ...ern. Cancer is a generic term for various neoplastic diseases that can occur many years after exposure to a carcinogenic substance. The best means of eliminating cancer is to define precisely the nature of the carcinogenic chemical, mixture of chemicals, or enhancing factors in the environment that produce the cancer (*1*). Bioassay studies in the early 1960s showed that environmental mixtures such as cigarette smoke and ambient air particulates can cause cancer in animals. Extensive studies were undertaken to determine the chemical components that could be responsible for such an effect. It was found that most environmental samples were chemically complex and comprised of thousands of chemical compounds (*2*, *3*). Identification of the most biologically active compounds presented an enormous if not an impossible task.

During the mid-1970s several microbial tests were developed that were simple, cheap, rapid, and above all genetically very well defined (*4*). The bacterial reverse mutation test battery in *Salmonella* as developed by Ames et al. (*5*) provided a vast body of information on the presence of genotoxic activity not only in defined industrial chemicals, pesticides, and cosmetics but also in complex mixtures such as air, water, and other complex sources of pollutants. Although this test, and others like it, were used to determine the relative mutagenic potency for a wide variety of environmental samples, such studies did not yield infor-

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Report

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mation on which specific compounds were primarily responsible for this activity.

The Ames tester strains were selected so that types of mutations (e.g., frame shift vs. base pair substitutions) can be distinguished. This information, together with differential responses (e.g., with and without microsomal activation), can be used to provide information about the general classes of chemicals causing the response. More recently, new tester strains that are sensitive to certain classes of chemical mutagens have been developed. For instance, strain TA98NR (Rosenkranz nitroreductase strain) is deficient in nitroreductase enzymes and therefore gives a reduced response to certain nitrated PAH compounds. The magnitude of results varies depending on the type of assay used, as shown for diesel particles in Table I. In addition, the contribution of an individual compound to total fraction mutagenicity is dependent on the type of bacteria used in the assay. These differences make it possible to apply these strains in a diagnostic way. Because strain TA98 has been used most frequently for assays of environmental samples, we will limit our discussion to its use in this REPORT.

It became apparent in the late 1970s that these bioassays could be used in combination with chemical fractionation to greatly simplify the process of identifying significant mutagens in complex environmental samples, such as diesel particles, petroleum and petroleum substitutes, chemicals in drinking water (*6*), and commercial products (*7*). The use of short-term bioassays in conjunction with analytical measurements constitutes a powerful tool for identifying environmental contaminants. We have coined the term "bioassay-directed chemical analysis" to best describe this marriage of analytical chemistry and biology. The objective of this methodology is to identify key compounds in various types of air pollutant samples. Once that task is completed, studies on metabolism, sources environmental exposure, and atmospheric chemistry can be undertaken.

Table I. Direct-Acting Mutagenicity of Diesel Particulate Extract (NBS SRM 1650)

Bacterial strain	Mutagenicity (rev/μg)
TA98	8.0
TA98NR	1.5
TA100	5.5
TA104	17.0

The protocol for bioassay-directed chemical analysis illustrated in Figure 1 was developed originally in our laboratories during the late 1970s (*8*, *9*) to identify chemical mutagens in diesel particulate extracts. At about the same time similar techniques for the identification of chemical mutagens in synthetic fuels were reported by Guerin et al. (*10*) and Wilson et al. (*11*) and in drinking water by Tabor et al. (*12*). More recently, these techniques have been used in a rigorous manner for the identification of mutagens in other types of air pollution samples, such as ambient air particulates, diesel particulates, and wood smoke (*13–15*), and in water pollution samples such as river water (*16*). Most of our work has been directed toward the characterization of ambient air and diesel particulates. We will use these examples to illustrate the analytical logic used in identifying the mutagenic components of complex mixtures.

Proper sampling, storage, and extraction of an environmental sample are crucial parts of the analysis. If the sample is not qualitatively and quantitatively representative of what is present in the environment, results obtained from the bioassay-directed chemical analyses are of little value in assessing environmental risk. However, it is not necessary to use a representative sample to develop methods. Some selected large samples collected in early studies were used for methods development. Since that time, the National Bureau of Standards (NBS) has made available a number of environmentally related reference materials that are useful in developing bioassay-directed chemical analysis techniques. These materials were not meant to be representative samples from which conclusions could be made concerning their origin or environmental risk. The major advantage of this approach is that a common and representative material is available to many laboratories for intercomparison of results. Two materials that have been used most widely for this purpose are NBS SRM 1650 (heavy-duty diesel particulates) and NBS SRM 1649 (ambient

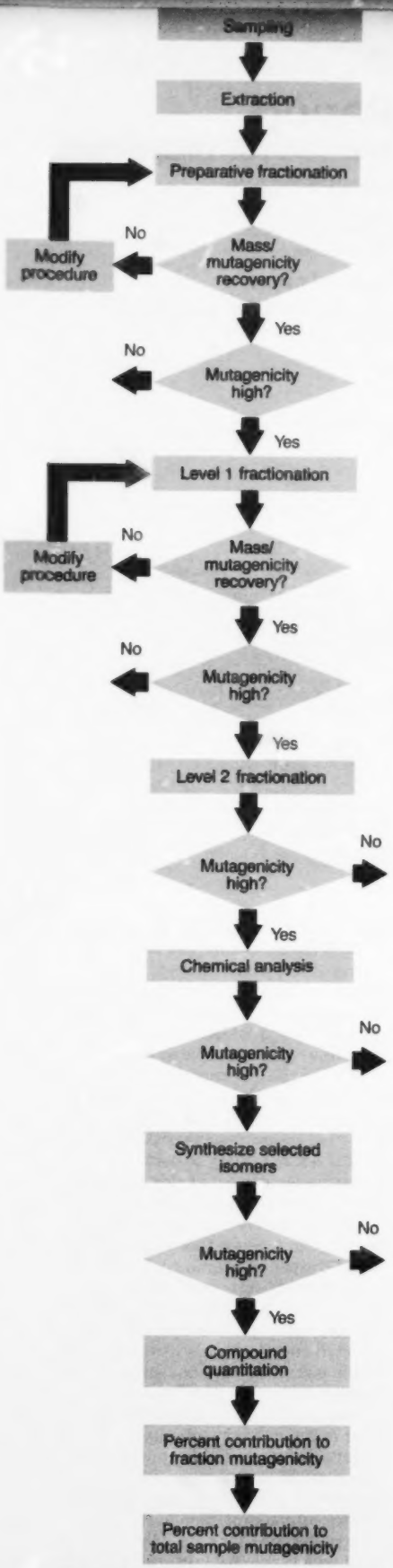

Figure 1. Protocol for bioassay-directed chemical analysis

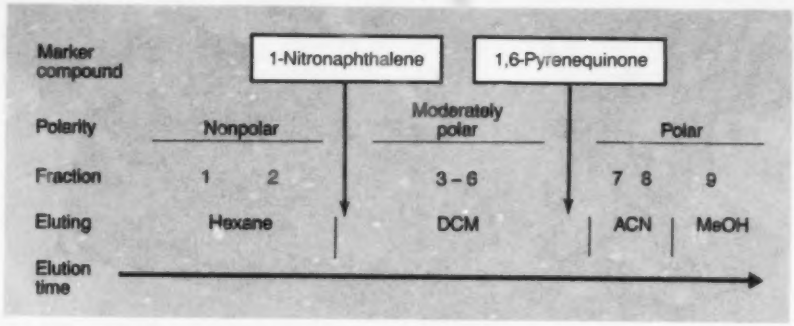

Figure 2. Designation of nonpolar, moderately polar, and polar fractions using normal-phase HPLC in the level 1 fractionation scheme

air particulate matter collected in Washington, D.C.).

Extraction

Sequential extraction with increasingly polar solvents, binary solvents, and supercritical fluid extraction are used to separate organic material from particulates and other types of solid environmental samples. A two-step extraction process using methylene chloride followed by methanol is effective for efficient recovery of mutagenicity and mass for several types of combustion emission samples. Liquid–liquid extraction using dichloromethane and diethyl ether is suitable for extraction of mutagenic material from heavily polluted waters. Adsorption of pollutants on resins followed by solvent elution is effective for concentrating organics that are present in low concentrations (*17*).

Preparative fractionation

A number of prefractionation and preseparation techniques have been developed to help simplify the analysis of environmental samples. They are usually applied on a preparative or semipreparative scale to yield gram to milligram quantities, respectively, of samples. The two most widely used techniques are chromatography on an open normal-phase silica column to separate groups of compounds on the basis of polarity and separation of compounds into acidic, basic, and neutral fractions.

Early attempts to chemically characterize the trace components in sample fractions proved to be extremely difficult, and it was estimated that hundreds of compounds were present. However, bioassay analysis helped researchers decide which fractions should be studied in more detail (Figure 1).

One of the concerns of the early work in the late 1970s was the potential loss and formation of mutagenic substances during analysis. The procedural recovery of mass and mutagenicity is determined by combining the individual fractions to produce a reconstituted sample. The mass and mutagenicity of this sample are then compared with that of the unfractionated sample. Another test is to determine if the biological activity of the unfractionated sample equals the sum of the activities of the individual fractions. If they are unequal, the mutagenicity may be due to synergistic or toxic effects or to the fact that cell killing was not taken into account. If the separation removes highly toxic components from the mutagenic components, the additivity of the fraction mutagenicities may be greater than 100%. Any bioassay that has enough sensitivity to obtain good quantitative results (*6*, *13*) on small (microgram) quantities of material can be used in the bioassay-directed chemical analysis technique.

Level 1 fractionation

Normal-phase high-performance liquid chromatography (HPLC) is a highly reproducible technique that separates environmental samples into chemical fractions of increasing polarity using hexane, dichloromethane (DCM), acetonitrile (ACN), and methanol (MeOH) as eluents (*8*). The reference materials 1-nitronaphthalene and 1,6-pyrenequinone may be used as chemical markers to designate the separation of samples into nonpolar (1–2), moderately polar (3–6), and polar (7–9) fractions (Figure 2). Results from a number of laboratories using slightly different fractionation procedures can be compared using this definition of polarity (*18*). It was found that the nonpolar fractions accounted for less than 2–3% of the total extract mutagenicity and that the distribution of moderately polar and polar materials was dependent on the sample source (Figure 3).

Table II gives the distribution of mass and mutagenicity for nonpolar, moderately polar, and polar fractions of NBS SRM 1650. Although 54% of the recovered mutagenicity is associated with polar compounds (fractions 7–9), the additivity of mass (94%) and mutagenicity (79%) is good. Diesel

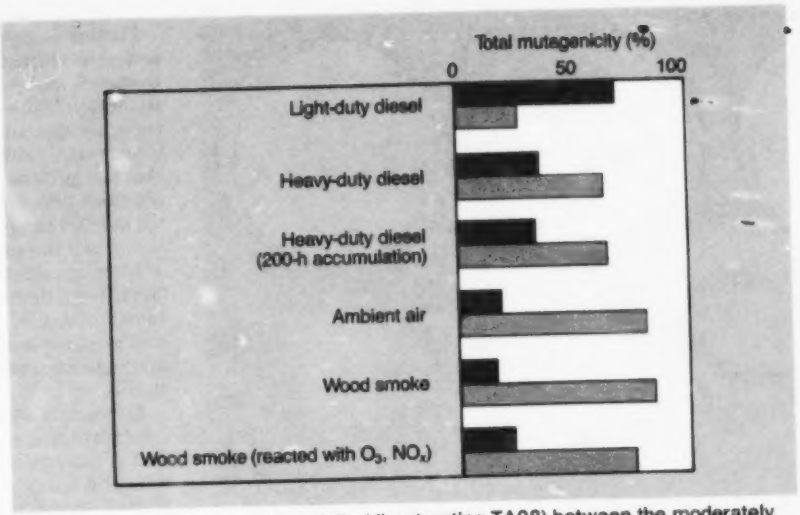

Figure 3. Distribution of mutagenicity (direct-acting TA98) between the moderately polar (green) and polar (blue) mutagenic fractions for six air particulate samples

particulate extract samples usually yield mass and mutagenicity additivities of greater than 90% and 70%, respectively, and mass and mutagenicity recoveries of better than 90%.

Chemical analysis

Studies were undertaken in the late 1970s to determine the composition of samples collected from the level 1 fractionation. High-resolution capillary column gas chromatography (GC) with selective detectors and coupled with mass spectrometry (GC/MS) proved to be powerful tools for the characterization of fractions.

By 1980 several laboratories, applying the techniques described so far, simultaneously made some important discoveries. Guerin et al. (*10*) and Wilson et al. (*11*) discovered the presence of highly mutagenic polynuclear aromatic amines (PAH–NH_2) in synfuels; Schuetzle et al. (*8*) found a significant mutagen, 1-nitropyrene, in diesel particles; and Lofroth et al. (*19*) and Rosenkranz et al. (*20*) identified 1,3-, 1,6-, and 1,8-dinitropyrenes as the major mutagenic substances in commercial carbon blacks. The amines and dinitropyrenes accounted for more than half of the sample mutagenicity in the case of the synfuels and carbon blacks, respectively. However, less than 25–30% of the mutagenicity of diesel particles could be accounted for by the 1-nitropyrene.

In the case of SRM 1650, GC and GC/MS analysis showed that hundreds of compounds were still present in each fraction. Figure 4 depicts a portion of a chromatogram generated

Table II. Mass and Mutagenicity Distribution (TA98, Direct Acting) for NBS SRM 1650[a]

Fraction	Distribution of mass (%)	Distribution of mutagenicity (%)
Nonpolar		
1	0.6	0
2	0.7	0
Moderately polar		
3	1.3	0
4	2.6	19
5	2.4	10
6	1.3	7
Polar		
7	3.7	24
8	41.6	9
9	39.9	10
Percent recovered	94.0	79

[a] Data from Reference 18

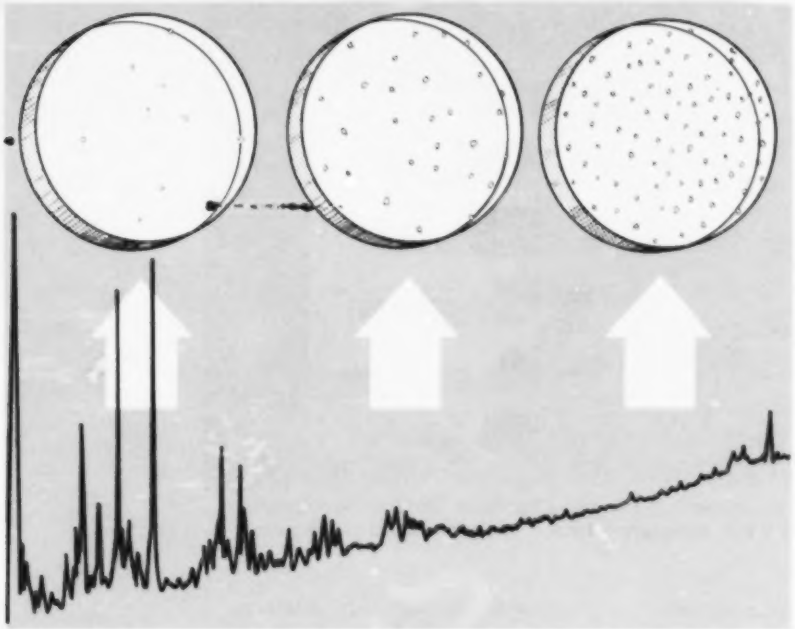

Figure 4. Capillary gas chromatogram of fraction 5 of a light-duty diesel sample with nitrogen-selective detection

Petri dishes at top illustrate results of Ames assays of subfractions of the diesel sample fraction

from the capillary column GC analysis of fraction No. 5 of a light-duty diesel sample using a nitrogen-selective detector. Scores of nitrogen compounds were found to be present in this fraction. GC/MS analysis verified that most of the nitrogen-containing compounds were nitrated polynuclear aromatic hydrocarbons (nitro PAHs). Ames assays of subfractions of fraction No. 5 showed that most of the mutagenicity was associated with compounds that eluted in the last one-third of the chromatogram.

Further analysis was made using direct-probe high-resolution mass spectrometry, high-resolution GC/high-resolution MS, and mass spectrometry/mass spectrometry (MS/MS). An important finding in these studies was that the moderately polar chemical fractions primarily consisted of substituted PAH compounds with ring sizes of 2–6 and the substituents consisted of hydroxy, aldehyde, anhydride, nitro, ketone, dinitro, and quinone functional groups (*8*). A potentially important group of mutagens, the nitrohydroxypyrenes, was identified in these studies (*21*).

Although a multitude of compounds were identified or tentatively identified, it was obvious that such fractions were still too complex to allow identification of chemical mutagens and that further separation of each fraction into subfractions would be necessary.

Level 2 fractionation

Further developments in HPLC, high-performance thin-layer chromatography (HPTLC) (*22*), and mutagenicity testing (*23*) have contributed to a much finer tuning of this combined chemical and biological approach to the characterization of mutagens in environmental samples. HPLC can generate scores of fractions, each of which is tested for mutagenicity. We refer to this resulting relationship of fraction mutagenicity to

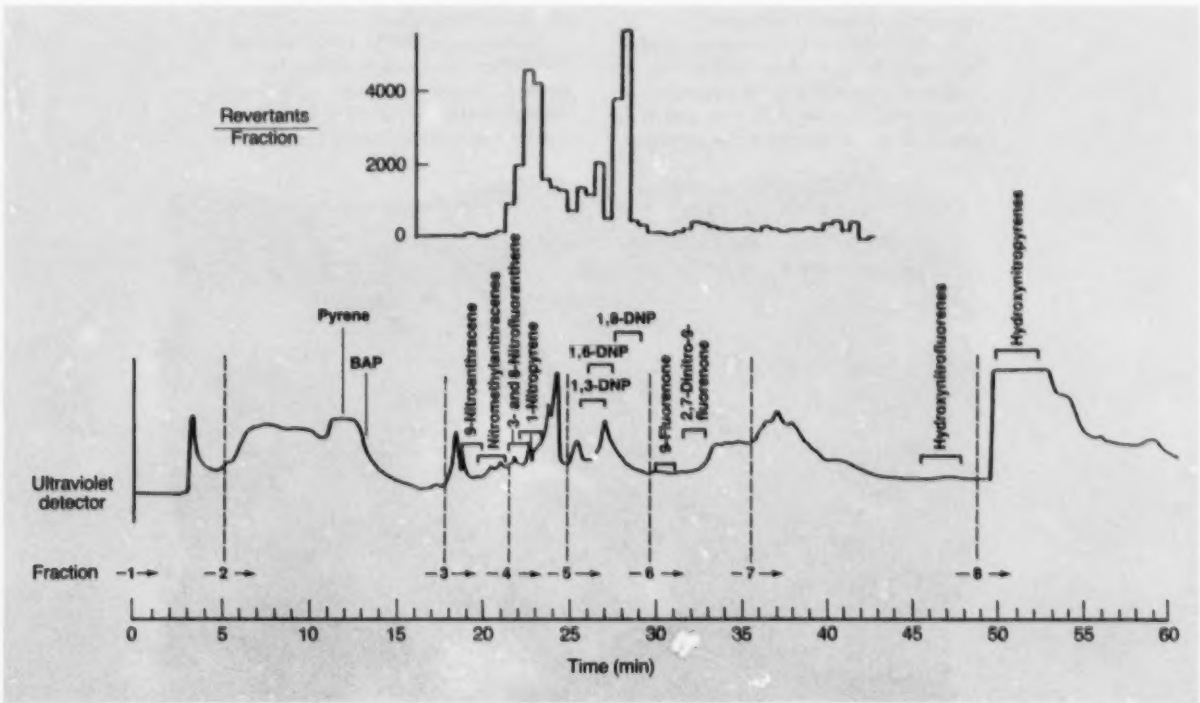

Figure 5. Ames assay chromatogram (TA98, without activation) and HPLC–UV analysis of a light-duty diesel particulate extract (level 1 fractionation)

Table III. Distribution of Mutagenicity and Mass for Acid, Base, and Neutral Subfractions of Polar Fractions 7, 8, and 9[a]

Subfraction	% distribution of mutagenicity (mass)		
	7	8	9
Acid	4 (27)	22 (39)	39 (70)
Base	19 (28)	14 (7)	4 (4)
Neutral	66 (59)	84 (41)	162 (26)
Total recovery	89 (114)	120 (87)	205 (100)

[a] Jensen, T.; Ball, J. Presented at the Symposium on the Chemical Characterization of Diesel Exhaust Emissions Workshop II, Coordinating Research Council, Dearborn, Mich., March 4–6, 1985.

fraction elution time as a bioassay chromatogram or an Ames assay chromatogram in cases in which the Ames test is used (*24*). A bioassay chromatogram for a diesel particulate sample is illustrated in Figure 5. The lower portion of the figure shows the corresponding HPLC chromatogram using a UV detector. The most mutagenic fractions have elution times that approximately coincide with 1-nitropyrene, 3-nitrofluoranthene, 8-nitrofluoranthene, 1,3-dinitropyrene, 1,6-dinitropyrene, 1,8-dinitropyrene, and 2,7-dinitro-9-fluorenone reference compounds.

GC/MS is a powerful technique for quantitative analysis of these trace constituents. Deuterated standards are added to the extracts before sample analysis, and quantitative results are readily obtained by comparing the signals from the eluting native and deuterated compounds. On the basis of such analysis it was found that 30–40% of the total recovered extract mutagenicity of SRM 1650 was attributable to the seven moderately polar nitro PAHs. The remainder of the mutagenicity (10–15%) was attributable to the presence of an unidentified compound or compounds eluting between 23 and 26 min; 30–35% was recovered in the polar fractions, and 5–10% was distributed about equally among the remaining fractions.

Although separation of the sample into acid, base, and neutral fractions is usually done in the early stages of sample preparation, this procedure can be used to further simplify the polar fractions. As an example, some preliminary data on the distribution of mutagenicity for the acid, base, and neutral fractions 7, 8, and 9 of SRM 1650 are given in Table III. This analysis shows that the most mutagenic compounds in the diesel sample are neutral in character. The additive mutagenicity for the acid, base, and neutral fractions is close to that of the unfractionated fraction 7. However, the additive mutagenicity for fractions 8 and 9 exceeded 100%. It is postulated that these high recoveries are indicative of chemical changes that may have occurred during acid–base extraction or separation of toxic compounds from these fractions. High-resolution mass spectrometric analysis of fractions 8 and 9 shows that organic esters and acids of PAHs and aliphatic compounds are major components. Acid or base hydrolysis of PAH esters could account for increased mutagenicity.

The specific fractionation scheme that most effectively separates the mutagens from the nonmutagens while minimizing the destruction or creation of mutagens varies with the types of complex mixture being analyzed. Diesel emissions from different sources will vary in the amount of specific components but the general composition is sufficiently consistent to develop one approach to the bioassay-directed chemical analysis, although several may be equally effective.

The scheme used to identify mutagens in synfuels has concentrated on preparative separative techniques because large quantities of materials were needed for extensive biological and chemical characterization studies. A relatively high level of mutagenic activity was found in an 800–850 °F distillation fraction of a synfuel sample (*10*). Nitrogen-containing compounds were separated on an alumina column using $CHCl_3$/ethanol. Level 1 fractionation was accomplished on a silicic acid column. GC/MS was used to identify PAH-amines as the major mutagenic species in the synfuels.

Ambient air particulates contain more chemical components than most other types of environmental samples. For this reason, the identification of mutagenic species in ambient air particulates has been more difficult than in diesel emissions. However, bioassay-directed chemical analysis procedures are being used to single out some potential candidate compounds.

LC and HPLC fractionation used in combination with acid–base–neutral separation appears to be an effective protocol for this purpose, as illustrated in Figure 6 (*25*). Good recovery of mass (98%) and mutagenicity (86%) for an extract of the NBS SRM 1649 air particulate sample was obtained by elution of five fractions on silica gel using hexane, hexane–benzene, methylene chloride, methanol, and acidic methanol (*26*).

SRM 1649 was used to develop the

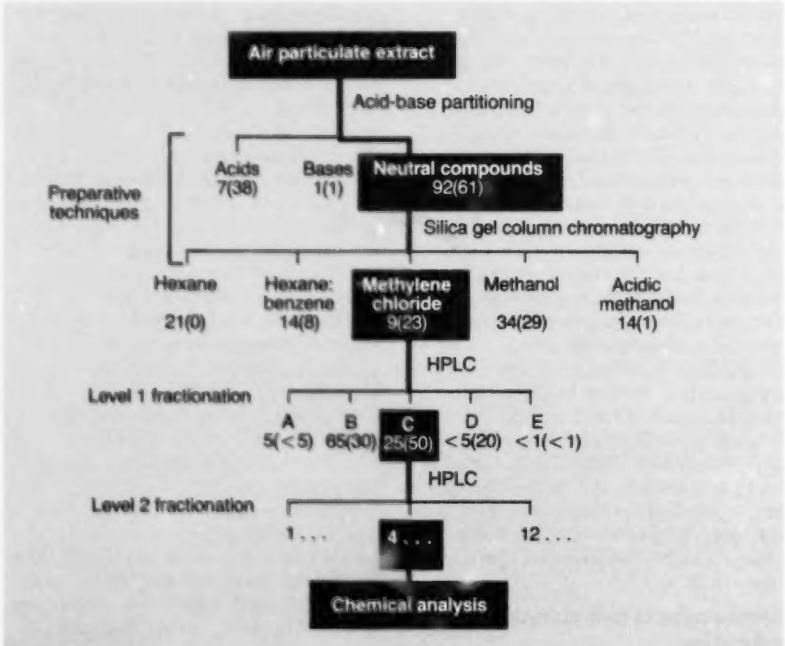

Figure 6. Bioassay-directed chemical analysis scheme for the analysis of NBS SRM 1649 (air particulate matter)

The numbers under each fraction represent the percentage distribution of mass and mutagenicity (in parentheses)

methodology for the ambient air samples. Once this methodology was developed, it was applied to fine air particles (<1.7 μm in diameter) collected in Philadelphia. The three fractions of greatest interest were the acid, methylene chloride, and methanol fractions, which produced 38%, 23%, and 29%, respectively, of the recovered mutagenicity (Figure 6). Because the methylene chloride fraction was more amenable to subfractionation by existing HPLC methods, this fraction was chosen for further fractionation. Normal-phase HPLC was used to fractionate the methylene chloride fraction (*26*).

The first HPLC subfractionation resulted in one fraction, methylene chloride-C, which contained nearly 50% of the mutagenicity in 25% of the mass. Analysis of this fraction by both electron impact and negative chemical ionization high-resolution (HR) GC/MS techniques indicated that the complexity of the sample was not reduced sufficiently to identify individual components. Subfraction C was further fractionated by normal-phase HPLC into 12 fractions for bioassay.

As we proceeded to higher levels of subfractionation, less and less mass was available for bioassay. In this study, the first-level fractions obtained from the preparative techniques were bioassayed in triplicate at seven doses with and without activation so that we could quantitate the distribution and recovery of mutagens. At the first and second levels of fractionation, fewer doses and plates were used for the bioassay until nearly the entire fraction was used for a single plate. Therefore, the ability to quantitate the recovery of both mass and mutagenicity decreases as we subfractionate. The bioassay chromatogram showed peaks of relatively higher mutagenicity in fractions 4 and 11 with 29 and 37 rev/μg, respectively. Mass spectral analysis of peak 4 demonstrated that the goal of sequential fractionation had been achieved such that individual components could be resolved and quantified using HRGC/MS. The major mutagenic compounds identified in the methylene chloride-C-4 fraction were hydroxy-nitro-substituted fluoranthenes (OH-NO_2-PAH) (*26*); work is continuing to synthesize and identify the specific isomers. This class of compounds (i.e., the hydroxynitropyrenes) also has been identified in diesel particulates (*21*, *27*).

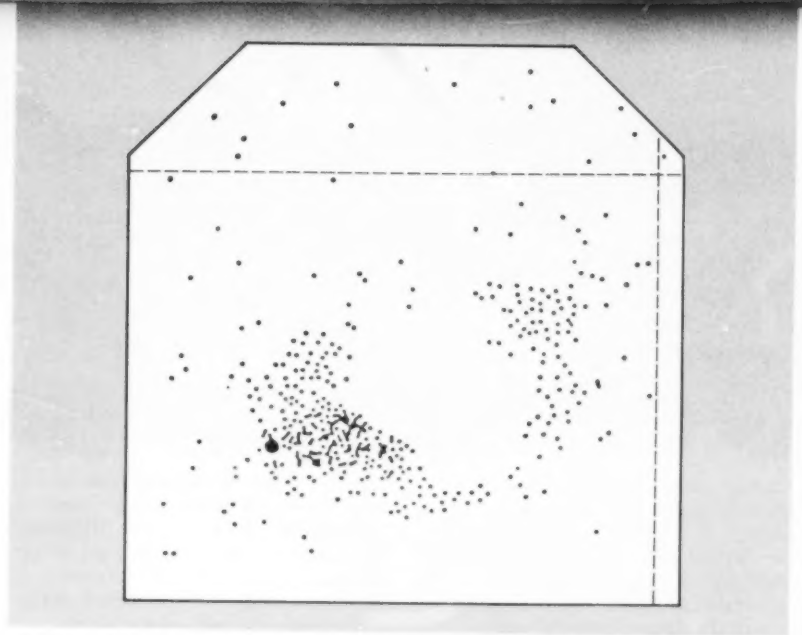

Figure 7. Distribution of mutagenicity for a combined Ames assay–HPTLC analysis of an ambient air particulate extract

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Development of new analytical techniques

Despite the considerable progress that has been made in determining moderately polar organics in environmental samples, there remain classes of compounds that have not yet been identified because of limitations in the analytical methods and detection systems.

There has been much less success with the determination of labile compounds (e.g., organic peroxides) and polar organic species. To date, only a limited number of compounds have been identified or tentatively identified as potentially important mutagenic species in the polar fractions of particulate extracts. This lack of information is the result of problems associated with determining polar PAH derivatives, including sample loss from physical adsorption, chemical interactions among components, and sample decomposition. Several laboratories are now developing and applying new methodologies for the determination of polar chemical mutagens.

Direct-insertion probe distillation coupled with HRMS of whole extracts can be used as a survey technique to determine the types of compounds present or in some cases to identify groups of isomeric compounds (e.g., nitropyrenes and nitrofluoranthenes) (*8*, *28*). This technique is also useful for thermally labile organics.

MS/MS coupled with direct-insertion probe introduction of sample yields a higher level of specificity than can be obtained with the HRMS technique (*29*). Although this method does not readily yield specific isomer information, it is a valuable screening tool with a high degree of compound specificity. Secondary ion mass spectrometry (SIMS) using ion and neutral atom beams also may be useful for determination of polar and nonvolatile organic substances in environmental samples (*18*).

The above techniques are good for screening analyses. However, chromatographic separation is needed for positive identification of specific isomers in highly complex fractions. The poor recoveries GC and GC/MS achieve in the identification of polar compounds illustrate the limitations of GC techniques. One of the most promising analytical techniques for the determination of polar PAH derivatives appears to be supercritical fluid chromatography (*30*) and HPLC coupled with mass spectrometry (*31*).

New micromutagenesis methodologies that permit the quantitation of mutagenic activity on microgram quantities of material can now be used together with analytical-scale reversed-phase HPLC. The micro-forward mutation assay, which measures both mutagenicity and survival (toxicity), provides potentially more quantitation of a single-fraction mutagenicity (*32*, *33*), whereas the micro-reverse suspension assay (*34*) uses the standard Ames and Rosenkranz strains. We have successfully coupled these assays to analytical reversed-phase HPLC separation and analysis of polar mammalian metabolites of nitropyrene. This approach shows great promise in helping to identify the polar mutagens in complex environmental samples.

Recently, techniques have been developed to couple liquid chromatography directly with bioassay analysis (*35*, *36*). Duplicate (HPTLC) plates are used to fractionate organic matter.

One plate is used for chemical class tests and the second plate is used for an in situ Ames bioassay. This system has revealed some surprising and interesting differences among particulate matter samples from different cities. Figure 7 shows the distribution of mutagenicity for a combined Ames assay–HPTLC analysis of an ambient air particulate extract. Although the compounds responsible for the concentrated areas of mutagenicity are not known, it is envisioned that future advances in chemical analysis will help this effort. One such aid would be direct chemical analysis of the HPTLC plates using ESCA or SIMS to generate spatial chemical maps of the surface, which could be correlated with the pattern of mutagenicity.

In the future, a substantial effort will be needed to determine the compounds responsible for the mutagenicity of air and water pollutants. Bioassay-directed chemical analysis will continue to be a valuable tool for this purpose.

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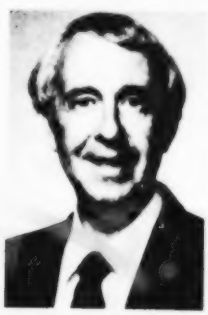

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